Aflatoxin contamination in cassava chips and assessment of Manihot esculenta crantz autodefence mechanism

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DEDICACE

In memory of **Dr Pascal Fandohan**, "*my father in the scientific world*", former director of the Program on Agricultural and Food Technology (PTAA), National Agricultural Research Centre of Agonkanmey (CRA-A), National Institute of Agricultural Research of Benin (INRAB).

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ABSTRACT

Scientific information about the aflatoxin status of cassava chips and *Manihot esculenta* Crantz autodefence mechanism against *Aspergillus* and thereby aflatoxin contamination is scarce. In this thesis we have established a new analytical method of aflatoxin determination in processed dried cassava root and investigated the fungal flora and the aflatoxin status of marketed cassava chips in Benin. Furthermore, we have studied *Manihot esculenta* Crantz autodefence mechanism through an *in vitro* test. All the above, were supported by comprehensive reviews on the importance and used of cassava or manioc as a major root crop to nourish people worlwide, on the aflatoxin contamination of food commodities and its implications in the developing world and on scopoletin as a coumarin phytoalexin with medical properties, respectively.

A new High Performance Liquid Chromatography (HPLC) – PHRED (photochemical reactor for enhanced detection) method for the analysis of aflatoxin in cassava flour was developed and validated. The method was validated for recovery, linearity, and precision at different concentrations. Recoveries ranged from 52 -70%, 69-85% and 80-89% at 5.0, 10.0, and 20.0 μg/kg, respectively. It would appear that the extraction step, i.e. amount of salt (NaCl) and shaking time are critical in this method which showed optimal performance when 1 g of salt was used and shaking was for 10 min. There was good linearity and precision and the separation allowed for baseline separation from interferences, such as coumarins.

Cassava chips marketed in Benin was characterized for their fungal flora. This was dominated by *Aspergillus* spp, notably *A. flavus*. Eighteen *A. flavus* isolates were characterized for the occurrence of the gene cluster *Nor1*, *Omt1* and *OmtB* responsible for aflatoxin biosynthesis using polymerase chain reaction method (PCR); 6 isolates were positive.

The HPLC – PHRED method was used to analyze for aflatoxin in marketed cassava chips from Benin. Interestingly, aflatoxin was not detected in any of the analyzed samples.

There was an accumulation of scopoletin, a coumarin compound in cassava varieties (*Kpaki kpika, kpaki soan, logoguesse kotorou* and BEN 86052) used in Benin for chips production. Investigation was performed by High Performance Liquid Chromatography (HPLC). Scopoletin was detected in all four varieties with a mean in fresh roots between 4.1-11.1 mg/kg dry weight. A strong increase in the content of scopolein was noticed after a peeling and drying process (6 days) for chips production, the mean content reaching 242.5 mg/kg dry weight in the variety BEN 86052. After three months of storage the content of scopoletin had decreased to 0.7 mg/kg dry weight.

An *in vitro* test was used to evaluate the inhibitory effect on the growth and aflatoxin production of one aflatoxin positive strain. While the growths were not significantly inhibited, aflatoxin production was inhibited by scopoletin at lower concentrations (0.024 mM). This compound could be used on other food commodities susceptible for mycotoxin contamination.

RÉSUMÉ

Des informations scientifiques au sujet du statut des cossettes de manioc (*Manihot esculenta* Crantz) par rapport à l'infection par *Aspergillus* spp et la contamination par les aflatoxines et le mecanisme d'autodéfence du manioc sont rares. Dans cette thèse nous avons mise au point et validée une nouvelle méthode analytique pour la détermination des aflatoxines dans les cossettes et avons identifié la flore fongique et quantifié les aflatoxines dans les cossettes de manioc vendues sur les principaux marchés au Bénin. De plus, nous avons également étudié le mécanisme d'autodéfence du manioc, ceci par un test *in vitro*. Tous ces travaux de recherche ont été soutenus par des revues complètes (1) sur l'importance et les utilisations du manioc, culture pour nourrir les populations du tiers monde, (2) sur la contamination des aflatoxines dans les produits agricoles et ses implications dans les pays en voie de développement et (3) sur la scopoletine comme une coumarine phytoalexine et ses propriétés médecinales.

La nouvelle technique d'extraction et le dosage [chromatographie liquide sous haute pression couplée avec le réacteur photochimique (HPLC-PHRED)] des aflatoxines dans la farine de manioc issue des cossettes de manioc est mise au point et validée. En effet, cette méthode a été validée pour les recouvrements, les coefficients de linéarité et la précision à différentes concentrations. Les taux de recouvrements varient respectivement entre 52 -70%, 69-85% et 80-89% pour les concentration d'aflatoxines de 5,0; 10,0; et 20,0 µg/kg. L'étape d'extraction notamment la quantité de sel (NaCl) a y ajouté et le temps de secouage sont critiques dans cette méthode qui a montré une extraction optimale avec 1 g de sel et un temps de secouage de 10 minutes. Les coefficients de linéarités et la précision obtenus sont très bonnes ce qui a permis la séparation des chromatogrammes qui pourraient présentés des interférences avec ceux des aflatoxines (example les coumarines).

Des échantillons de cossettes de manioc vendues sur les principaux marchés du Bénin ont été évaluées par rapport à leur infection par la flore fongique. Les espèces du genre *Aspergillus*, notamment *Aspergillus flavus* sont les plus rencontrées. Dix-huit (18) souches de *A. flavus* ont été caractérisées pour la présence des gènes *Nor1, Omt1* et *OmtB* qui sont les principaux gènes du cluster responsable de la biosynthèse des aflatoxines suivant la méthode de réaction par polymérisation et amplification des

gènes (PCR); six (6) souches sont positives c'est-à-dire possèdent simultanément les trois gènes du cluster.

La méthode HPLC-PHRED a été utilisée pour analyser les aflatoxines dans les cossettes de manioc échantillonnées sur les principaux marchés du pays. Il ressort que les aflatoxines ne sont pas détectables donc absentes dans les cossettes de manioc analysées.

Par ailleurs, une analyse de la concentration des composées coumarines notamment la scopoletine a été réalisée utilisant la chromatographie liquide sous haute pression (HPLC) sur des racines fraîches et cossettes issues des principales variétés de manioc (*kpika de Kpaki, kpaki soan,* logoguesse kotorou et BEN 86052) utilisées pour la fabrication des cossettes de manioc. La scopoletin est détectée dans chacune des quatre variétés avec une moyenne dans les racines fraîches variant entre 4,1-11,1 mg/kg de poids sec. Une augmentation significative de cette teneur en scopoletine est notée après épluchage et séchage des cossettes qui a duré 6 jours avec une teneur moyenne de 242,5 mg/kg de poids sec dans les cossettes issues de la variété BEN 86052. Après trois (3) mois de stockage cette teneur en scopoletine a diminué significativement pour atteindre 0,7 mg/kg de poids sec.

Le test *in vitro* a été utilisée pour évaluer l'effet inhibiteur de la scopoletine sur la croissance ou développement de *A. flavus* et la production des aflatoxines par les souches positives. Tandis que la croissance n'était pas sensiblement affectée, la production des aflatoxines est inhibée même aux concentrations inférieures (0,024 mM). La scopoletine pourrait donc être utilisée sur d'autres produits agricoles très susceptibles à la contamination par les mycotoxines.

RESUMÈ

Der er for nærværende en meget begrænset dokumenteret viden om aflatoxin niveauet i chips fremstillet af manjok (cassava) rod (rod af *Manihot esculenta* Crantz). Også den publicerede forskning vedrørerende plantens forsvarsmekanismer mod *Aspergillus* svampe og dermed kontaminering med aflatoxin er begrænset. Nærværende afhandling beskriver udviklingen af en ny analytisk kemisk metode til bestemmelse of aflatoxiner i tørret forarbejdet manjokrod og gør rede for resultaterne af en undersøgelse af svampefloraen og aflatoxin inholdet på markedsførte manjok chips indsamlet i Benin. Endvidere har vi under anvendelse af en in vitro tests studeret Manihot esculenta's kemiske forsvarsmekanisme mod svampe infektion/dannelse af mykotoksiner. Resultaterne af de nævnte undersøgelser præsenteres i originalartikler og perspektiveres af omfattende reviews om emnerne (1) manjoks betydning som afgrøde på verdensplan, (2) aflatoxin kontaminering af fødevarer og dennes betydning for udviklingslandes fødevaresikkerhed samt (3) scopoletin som en phytoalexin med coumarin struktur dens virkning for planten og dens medicinske egenskaber.

En ny High Performance Liquid Chromatography (HPLC) – PHRED (photochemical reactor for enhanced detection) metode til analyse af indholdet af aflatoxin I manjokmel (fremstillet af manjok chips) blev udviklet og valideret. Valideringen omfattede genfindelse (recovery), linearitet og præcision ved forskellige koncentrationer. Genfindelsen i procent varierede fra 52 -70 over 69-85 til 80-89% ved henholdsvis 5.0, 10.0 og 20.0 μg/kg. Ekstraktionen som bl.a. omfatter tilsætning af salt (NaCl) viste sig at være den mest kritiske fase med optimalt resultat ved tilsætning af 1g og omrystning i 10 minutter. Lineariteten var god og den ny metode tillod basislinje adskillelse fra intefererende stoffer; heriblandt coumariner.

Manjok chips indkøbtpå markeder spredt over Benin blev som nævnt analyseret for deres svampeflora. Denne domineredes af *Aspergillus* spp. Med *A. flavus* som den vigtigste. Atten isolater af *A. flavus* blev under anvendelse af polymerase chain reaction method (PCR) undersøgt for forekomsten af de tre gener *Nor1, Omt1* og *OmtB*. Disse er tilsammen ansvarlige (nødvendig) for en aktiv biosyntese af aflatix(er). Seks af de atten isolater viste sig at være positive for alle tre gener.

Den udviklede HPLC – PHRED method blev anvendt til at analysere de indkøbte chips for forekomsten af aflatoksiner. Alle chips prøver viste sig ikke at være kontaminerede (eller at være kontaminerede under detektionsgrænsen).

Rødder af de fire manjok varieteter *Kpaki kpika, kpaki soan, logoguesse kotorou* og BEN 86052, som alle i Benin anvendes til fremstilling af chips, blev under anvendelse af HPLC undersøgt for deres indhold af coumarinen scopoletin. Scopoletin blev påvist i friske rødder fra alle fire varieteter med et gennemsnits indhold som varierede fra 4,1 til 11,1 mg/kg tør vægt mellem varieteterne.
Fremstilling af chips indbefatter skrælning af de friske rødder og efterfølgende tørring i 6 dage. Denne behandling medførte en kraftig stigning i indholdet af scopoletin, som for varieteten BEN 86052 i gennemsnit kom op på 242,5 mg/kg tør vægt. Efter tre måneder på lager var dette indhold atter faldet til 0.7 mg/kg tør vægt.

Et af de 6 isolater af A. flavus som var fundet at besidde generne *Nor1, Omt1* og *OmtB* blev anvendt i en såkaldt *in vitro* test for indflydelsen af (1) mel fremstillet af chips fra manjokrod, (2) ekstrakter heraf og (3) ren scopoletin på svampens vækst hhv dannelse af aflatoxin(er). Mens væksten ikke påvirkedes nævneværdigt inhiberede alle tre komponenter syntesen af aflatoxin. Eksperimenter under anvendelse af manjok mel viste total inhibering ned til et niveau svarende til (0.024 mM).

LIST OF ABBREVIATIONS

^{o}C	: degree celcius
g	: gramme
µg/ml	: microgramme per milliliter
µg/kg	: microgramme per kilogramme
mg/kg	: milligramme per kilogramme
nmol/g	: nanomolar/gramme
%	: percentage

All other abbreviations used in this thesis are defined when first used.

LIST OF PAPERS

I. G.J. Benoit Gnonlonfin, Ambaliou Sanni, Leon Brimer. Preservation of Cassava (*Manihot esculenta* Crantz): a major crop to nourish people worldwide. Accepted in 2010 as a chapter in book "Progress in Food Preservation" *Wiley Blackwell* (in press).

II. G.J. B. Gnonlonfin, K. Hell, P. Fandohan, C.S.Y. Adjovi, D.O. Koudande, G.A. Mensah, A. Sanni and L. Brimer. A review on aflatoxin contamination and its implications in the developing world: A Sub-Saharan African perspective. Accepted for publication in *Critical Reviews in Food Science and Nutrition* (in press). Article ID: 535718 (BFSN-2010-0143.R1).

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V. G.J. Benoit Gnonlonfin, David R. Katerere, C.S.Yann Adjovi , Leon Brimer, Gordon S. Shephard and Ambaliou Sanni. (2010). Determination of aflatoxin in processed dried cassava root: Validation of new analytical method for cassava flour. *Journal of AOAC International* 93 (6), 1882-1887.

VI. G.J.B. Gnonlonfin C.S.Y. Adjovi, D.R. Katerere, G.S. Shephard, A. Sanni, and L. Brimer Mycoflora and absence of aflatoxin contamination of commercialized cassava chips in Benin, West Africa. Submitted to *International Journal of Food Microbiology*.

VII. G.J.B. Gnonlonfin, F. Gbaguidi, J. Gbenou, A. Sanni and L. Brimer. Changes in scopoletin concentration in cassava chips from four varieties during storage. Submitted to *Journal of the Science in Food and Agriculture*.

VIII. G.J.B. Gnonlonfin, Y. Adjovi, F. Gbaguidi, J. Gbenou, L. Brimer and A. Sanni. Scopoletin in cassava products as an inhibitor of aflatoxin production. Submitted to *Journal of Food Safety*.

Others papers from the author related to the work, but not included in this thesis

IX. G.J.B. Gnonlonfin, K. Hell, P. Fandohan, A.B. Siame. (2008). Mycoflora and occurrence of aflatoxins and fumonisin B_1 in cassava and yam chips from Benin, West Africa. *International Journal of Food Microbiology* 122, 140-147.

X. G.J.B. Gnonlonfin, K. Hell, A.B. Siame, P. Fandohan (2008). Infestation and population dynamics of insects on stored cassava and yam chips in Benin, West Africa. *Journal of Economic Entomology* 101, 1967-1973.

Others papers from the author, not included in this thesis

I. K. Hell, **B.G.J. Gnonlonfin**, G. Kodjogbe, Y. Lamboni, I.K. Abdourhamane. (2009). Mycoflora and occurrence of aflatoxin in dried vegetables in Benin, Mali and Togo,West Africa. *International Journal of Food Microbiology* 135, 99-104.

II. P. Fandohan, **B. Gnonlonfin**, A. Laleye, J.D. Gbenou, R. Darboux and M. Moudachirou. (2008). Toxicity and gastric tolerance of essential oils from *Cymbopogon citratus*, *Ocimum gratissimum* and *Ocimum basilicum* in wistar rats. *Food and Chemical Toxicology* 46, 2493-2497.

III. P. Fandohan, **B. Gnonlonfin**, K. Hell, W.F.O. Marasas, and M.J. Wingfield. (2006). Impact of indigenous system and insects' infestation on the contamination of maize with fumonisins. *African Journal of Biotechnology* 5 (7), 546-552.

IV. P. Fandohan, **B. Gnonlonfin**, K. Hell, W.F.O. Marasas, and M.J. Wingfield. (2005). Natural occurrence of *Fusarium* and subsequent fumonisin contamination in preharvest and stored maize in Benin. *International Journal of Food Microbiology* 99, 173-183.

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VI. G.J.B. Gnonlonfin, Adeoti R., Fanou L., Agnila B., Coulibaly O., Fandohan P., Hell K., Bessy C., Koudandé D.O. et Mensah G. A. (2010). « Good agricultural practices for cashew nuts production intended for export: harvesting, drying, storage and packaging». Bonnes pratiques de récolte, séchage et stockage de noix brutes d'anacarde destinées à l'exportation. ISBN: 978-99919-365-9-8, 15 p.

VII. Gnonlonfin G.J.B., Fanou L., Nanoukon M., Klotoé A., Houssou P., Fandohan P., Adeoti R., Coulibaly O. et Mensah G. A., (2010). «Good agricultural practices for cashew nuts production intended for export: harvesting, drying, storage and packaging». Framers training module. 27 slides. Bonnes pratiques de récolte, séchage et stockage de noix brutes d'anacarde destinées à l'exportation. Modules de formation. ISBN: 978-99919-365-8-1, 27 diapositives.

VIII. P. Fandohan, **G.J.B. Gnonlonfin**. (2007). «Guidance for mycotoxin reduction in maize during storage period». Guidance to be followed to reduce mycotoxin contamination of maize during storage «Guide à suivre pour réduire la contamination du maïs par les mycotoxines pendant le stockage».

IX. P. Fandohan, **G.J.B. Gnonlonfin**. (2007). «Utilization of processing methods for mycotoxin reduction in maize based food». Utilisation de processus de transformation pour la réduction du niveau de mycotoxine dans les aliments à base de maïs.

INTRODUCTION

The increasing worldwide concern about food safety has enhanced interest in fungal infection and subsequent production of mycotoxins in food products. Food safety can also be deteriorated by insects. Cassava (*Manihot esculanta* Crantz subspecies *esculanta*) is one of the most important dietary staple foods in many countries in the world (FAO 2002). It is an important starchy root crop, eaten and used by millions of people in West Africa, parts of East and Central Africa and elsewhere in the tropical world. In Benin, cassava is one of the most important crops with about 2.7 million tones produced in 2007 (FAO, 2008). The production of cassava is hindered by storage problems due to the high perishibility of the root and its susceptibility to contamination by fungi (Ikotun, 1989) and bacteria (Ikotun, 1983) as well as to sprouting due to increased metabolic activity (Ugochukwu et al., 1974). Cassava thus requires processing to ensure stability during storage. One way is to process tubers into dried cassava chips (Bricas et al., 1997). The processing of cassava into chips is a traditional activity in Benin during the dry season, especially in the Central and Northern regions of the country. Cassava chips when made into flour constitute a staple food for the majority of the people in Benin. The flour can be cooked alone or mixed with maize flour to make "dough". Cassava flour is also used in the baking process.

Cassava chips are subject to attacks by fungi of which the most important genera are *Aspergillus, Fusarium, Penicillum* and *Mucor* (Wareing et al., 2001; Bassa et al., 2001, Gnonlonfin et al., 2008). Fungal contamination can lead to the discolouration of the chips, mouldy taste, production of off odours (Gwinner et al., 1996) and possibly the production of toxins that are harmful to animals and humans (Sajise and Ilag, 1987). Even at very low quantities (parts per billion) this contamination can cause a significant effect on food safety and economic value of affected crops (Campbell et al., 2003).

Previous works have revealed the absence of aflatoxins in cassava chips produced in the rural areas in Ghana. However, other mycotoxins including sterigmatocystin, patulin, cyclopiazonic acid, penicillic acid and tenuazonic acid have been reported on samples of cassava chips collected in Ghana (Wareing et al., 2001). Similar work in Benin has also

revealed the absence of aflatoxin in cassava chips produced in the rural areas even though *Aspergillus flavus*, fungus responsible for aflatoxin production was present (Gnonlonfin et al., 2008).

Cassava chips produced in the rural areas are taken to the market for sale. Chips are stored in different ways by farmers, wholesalers and retailers. In the farmers' house, chips are stored in bulk on the floor (cemented or uncemented) while storage in woven polyethylene bags in the warehouse are common in the market. Some of these variations in storage conditions may promote fungi proliferation and subsequent toxin production. Therefore there is a need to assess cassava chips for sale in the market for aflatoxin contamination before a definitive statement can be made on the safety of the food product. Different analytical methods exist for aflatoxin determination in food (Trucksess and Pohland, 2002; Shephard, 2008). However, there is no validated method for aflatoxin analysis in cassava flour matrix made from chips.

The previous work conducted in Benin showed that high levels of *Aspergillus* infection and subsequent aflatoxins contamination of maize were associated with warmer and drier climates (Hell et al., 2003). Setamou et al. (1997) showed different and quite high levels of aflatoxins in maize samples collected from Northern Guinea Savannah (80.6 µg/kg) and Sudan Savannah (27.5 µg/kg). In these agroecological zones maize is grown as well as cassava chips are produced and sometimes stored together with maize. Therefore, cross contamination by toxigenic *A. flavus* can occur.

On the other hand in the Northern Guinea Savannah, it was also very common to observe that chips were left to dry along the roadside or on the roof and when dry still left there till used. Chips which are big in size and thicker, take long time for drying (about 20 days). Under these conditions chips are wettened with dew over the night especially during the period also showing hazardous rain the so-called "harmattan" period. They are re-dried again during the day when the sun is shinny with the increasing of temperature. A cycle of environmental condition is then established during the drying period. This variation could influence the infection rate.

A. flavus species that is ubiquitous in warm tropical environments is associated with cassava chips contamination (Egel et al., 1994; Gnonlonfin et al., 2008). The fungus *A. flavus* is a highly diverse asexual species that can be divided on the basis of physiological, morphological, and genetic criteria. The polymerase chain reaction (PCR) is one of the molecular technique useful for DNA isolation and amplification of it specific regions (Vosberg, 1989). However, most crop contamination is apparently caused by either the S or the L strains of *A. flavus* (Cotty and Cardwell, 1999) mixture being seeing less frequently. The S-strain isolates produce significantly higher levels of aflatoxins than L-strain isolates. In some agricultural regions, S-strain isolates dominate and are responsible for most of the aflatoxin-producing potential of the resident *A. flavus* communities (Orum et al., 1999; Horn and Dorner, 1999). However, not all *A. flavus* strains can produce aflatoxin and even those that can, do not do so under all conditions. So far, information on the molecular characteristics of fungus revealed on cassava has not been provided.

It has been reported that anti-microbial and fungitoxic compounds such as scopoletin, a coumarin compound, can accumulate in roots and tubers as a result of post-harvest physiological deterioration (Buschmann et al., 2000; Rodriguez et al., 2000; Gomez-Vasquez et al., 2004). The possible antimicrobial functions of scopoletin isolated from cassava against a variety of different fungal species have been mentioned (Rodriguez et al., 2000). According to this hypothesis this compound may negatively influence fungal growth and could explain the absence of mycotoxin in cassava (Gomez-Vasquez et al., 2004).

OBJECTIVES OF THIS STUDY

Taken all the above in consideration, it was important to carry out further studies in order to ensure the quality of cassava product with main focus on chips, recommend technologies to minimize storage loss and to increase utilization.

In general, this work aimed at enhancing chemical safety of cassava food products such as cassava chips, flour and lafun (fermented cassava by-product) with regard to lowering mycotoxin contamination by the use of biological control.

Specifically, this work aimed at:

- evaluating mycotoxin quality of cassava chips,
- molecular characterization the *A. flavus* isolated and,
- assessing the cassava (*Manihot esculenta* Crantz) autodefence mechanisms.

This thesis is based on the following papers referred to in the text by their Roman numerical.

Paper I. G.J. Benoit Gnonlonfin, Ambaliou Sanni, Leon Brimer. Preservation of Cassava (*Manihot esculenta* Crantz): a major crop to nourish people worldwide. Accepted in 2010 as a chapter in the book "Progress in Food Preservation" *Wiley Blackwell* (in press).

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MAIN RESULTS AND DISCUSSION

There is an increasing worldwide concern about food safety that has enhanced also interest in fungal infection and subsequent production of mycotoxin. Cassava or manioc is one of the most important staple foods in many developing countries but not much attention has been given to microbial and toxin contamination and to the defence mechanisms of this plant and its products.

This work contains 8 scientific papers on the area of fungal infection, aflatoxin contamination and autodefence mechanisms. Each paper treats specific aspects. A comprehensive review paper on the importance of the cassava root crop in the developing world (Paper I) followed by aflatoxin contamination and its implications in the developing world : A Sub-Saharan African perspective and Scopoletin a coumarin phytoalexin with medical properties, paper II and paper III, respectively. These reviews papers highlight all aspects on each of the topics including some perspectives relevant to both developing and devolped countries. Paper IV starts by looking at the cassava varieties used for chips production, their characteristics and farmers' perceptions of the potential toxicity of raw cassava roots. Paper V addresses the issue of a new analytical method for aflatoxin determination in cassava flour made from chips. Paper VI looks closer to the fungal flora and aflatoxin contamination in commercialized cassava chips in Benin using the new analytical method. While papers VII and VIII addresses the accumulation of scopoletin in cassava varieties used for chips production and scopoletin as an inhibitor of aflatoxin production, respectively.

A survey has been conducted in the frame time of this work and farmers have been interviewed on number of varieties/cultivars in use, origin of these cultivars, main activities of the respondent, test for roots toxicity and the end use of chips (Paper IV). It appears that cassava chips production is carried out solely by women and most of them (85%) are not educated. This observation raises the question of education which can be a factor for food safety. Furthermore, the importance of cassava chips production in the diet and for income generation of farmers in the household sampled is proven. Farmers

used attributes of taste to describe cassava. The practice of tasting the tuberous roots to evaluate the need for processing (degree of bitterness as associated with toxicity) was carried out by women and men, but to varying degrees (Paper IV). Nyirenda et al. (2011) have demonstrated that farmers also use taste to describe cassava varieties in Malawi and Zambia. Furthermore, this work shows that certain morphological characteristic including leaf shape or colour (Paper IV) can also be used to group cassava varieties as reported by early works (Chiwona-Karltum, 2001; Mkumbira et al., 2003). In contrast, these morphological characteristics are not used by farmers in Nigeria and Tanzania for this purpose (Oluwole et al., 2007).

From an earlier work we have reported the absence of aflatoxin in cassava chips processed and stored in farmers' household (Gnonlonfin et al., 2008). Paper VI confirms this finding in marketed cassava chips with the help of the new analytical method (Paper V) even though *A. flavus* was present and the storage conditions differs from farmers' household to market level.

We have also investigated the accumulation and subsequent changes in concentration of scopoletin, a coumarin compound in the four cassava varieties (*Kpaki kpika*, *Kpaki soan*, *Logo guesse kotorou* and BEN 86052) during storage (Paper VII). Early work has reported the accumulation of scopoletin in colombian cassava cultivars that have been stored as fresh cutted root slices under controlled conditions (dark, 29^{o}C and 80-90% relative humidity) for 6 days (highest content of scopoletin reached 123.94 nmol/g fresh weight) (Buschmann et al., 2000). The present investigation is first report on processed cassava chips that was sun drying for 6 days at environmental conditions (mean temperature of 33^{o}C and relative humidity of 34.6%) (Paper VII). This processing method helps to prolonge the shelf life of the product which is a staple food for many people especially in the developing world. On the other hand the biosynthesis of hydroxycoumarins and the consequences is still not fully clear. For scopoletin, a separate synthesis from ferrulic acid was suggested by Strack (1997). In contrast, Cabello-Hurtado et al. (1998) suggest a synthesis starting from caffeic acid to esculetin which then is

modified to scopoletin. It seems that there are different possible pathways and that plants may have developed convergent pathways leading to scopoletin (Kai et al. 2006).

Different biological activities of scopoletin have been described (Rodriguez et al. 2000). However, this is the first time that scopoletin is investigated against *A. flavus* growth and aflatoxin production (Paper VIII). Scopoletin seems to act as an aflatoxin synthesis inhibitor in cassava enriched media even at a lower concentration of 0.3 µg/ml (0.024 mM) using a toxigenic *A. flavus* strain (possesses genes of the cluster *Nor1*, *Omt1* and *OmtB*) (Paper VIII). The absence of fluorescence in the *in vitro* assay and the absence of aflatoxin in the previously analyzed cassava chips samples collected in Benin (Gnonlonfin et al., 2008; 2011), Tanzania (Muzanila, 2000), Ghana (Wareing et al., 2001) and Nigeria (Jimoh and Kolapo, 2008) can then be explained by an inhibition of aflatoxin synthesis. These results suggest that scopoletin could be used on other food commodities susceptible for mycotoxin contamination.

MAIN CONTRIBUTION AND PERSPECTIVES

In this study, we established and validated a new analytical method for aflatoxin determination in a complex matrix like cassava flour made from chips. This has highly contributed to the field of analytical methods used so far for aflatoxin determination in food. We have also shown that cassava chips marketed in Benin are not contaminated by aflatoxin using the newly established analytical method. However, there is still the need for more awareness of hygienic/sanitation and good drying and storage practices for cassava chips production. Thus, ensuring the safety of the product from all point of view.

We have also demonstrated the scopoletin accumulation, its decreasing level during storage and finally its inhibitory effect on aflatoxin production. These results call for more investigations especially:

- *in vivo* studies on other food commodities susceptible to fungal infection and mycotoxin contamination,
- studies to fully understand the mechanism behind the inhibitory effect of scopoletin and,
- studies to fully understand the biosynthesis of scopoletin.

These research works would add valuable information to mycotoxin reduction in contaminated food, thus ensuring the safety of the food.

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